Developing a protocol for the use of non-invasive genetic sampling to monitor UK red fox abundance

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David George Whittle

Abstract

The red fox is an important UK predator and is widely managed, due to its impact on species of economic and conservation concern. However, UK fox populations are currently poorly monitored, and population size estimates are almost exclusively from index counts such as the national game bag census. Estimates of true abundance could improve the monitoring and management of UK foxes by reducing the levels of uncertainty in population estimates.

Non-invasive genetic sampling (NGS) has a lot of potential as a method for estimating the true abundance of foxes, and this study trialled hair collection and analysis techniques for use in NGS studies of UK foxes. Several trap designs, incorporating different baits and hair collection structures, were set up in Durham City woodland and trialled for their effectiveness in attracting foxes and collecting hair samples. The traps differed in their effectiveness at collecting hair, but neither food baits nor valerian oil were successful in attracting foxes to traps. Further research is needed to determine a reliable method for collecting hair samples.

The Chelex extraction method was tested for its reliability for extracting DNA from single-hair samples, and was found to be extremely reliable for this purpose. A range of microsatellite markers were then tested for use identifying individual red foxes from hair samples, and a useable set of primers was identified, and optimised. A sex-linked marker (SRY) was also tested and optimised, to provide additional information at from samples. The resulting protocol was also tested with domestic dog samples, and the results of the analysis were found to be genetically distinct, showing that mistakenly included samples from closely-related species could be identified.

The developed laboratory methods could be reliably used for individual recognition and sex identification from remotely-collected hair samples from red fox populations in the UK, and could form a basis for future capture-mark-recapture and population analysis of red fox samples, improving red fox monitoring in the UK.

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Introduction

Natural wildlife provides a variety of benefits to humans. These can include economic gain and recreational enjoyment. These benefits can result indirectly from animal activity (e.g. Klatt *et al.*, 2013), as well as through exploitative activities, such as hunting (Brashares *et al.,* 2011). However, the conservation of wildlife can often conflict with human interests (Geist, 1994). For example, activities such as hunting and fishing can lead to overexploitation (Nielsen, 2006) and even the persecution of common pest species can have cascading effects which can result in biodiversity loss and loss of ecosystem function (Gaston and Fuller, 2008).

Wildlife conservation and management aim to address these human-wildlife conflicts, by using biological knowledge to reduce the impact of mankind on ecosystems (Festa-Bianchet and Apollonio, 2003). In particular, wildlife management aims to balance economics and other anthropocentric concerns with conservation and ecological issues, aiming to ensure that any exploitation or culling is ecologically sustainable and to minimise the loss of ecosystem function and biodiversity. In addition, wildlife management schemes can reduce the populations of invasive species (Marlow *et al.*, 2015) or other ecologically-damaging wildlife (Côté *et al.,* 2004), in order to protect both economic and ecological interests.

In order to determine acceptable management levels, wildlife management projects require accurate ecological data for the target populations, both before implementing management, and afterwards, in order to monitor its effects. Without adequate understanding of the ecology of the target species, management schemes may be ineffective at controlling pests (Newsome *et al.*, 2014), result in unintended negative effects (Bielby *et al.*, 2014), or even target species incorrectly (Gerber *et al*., 2009). Monitoring wildlife populations is therefore a highly important aspect of wildlife management, in order for these issues to be identified, and for developing improved procedures.

In Europe, the majority of population estimates for mammal species are based on indirect abundance measurements such as scat counts or detection frequency surveys, rather than surveys that estimate the true abundance of the population. These indirect counts are known as index sampling, which includes national surveys, such as the UK's national game bag census (Davey *et al*, 2010), as well as more local population estimates for local management and research purposes (e.g. Newsome *et al.*, 2014; Baker and Harris, 2006). Index surveys can vary greatly in methodology, ranging from highly invasive counts of the number of individuals taken by hunting (Davey *et al.*, 2010), through to non-invasive methods such as scat counts (e.g. Baker and Harris, 2006). However, the indices produced through all of these methods depend on the rate of encounters between the

observer (hunter, researcher, camera, etc.) and the target animals or signs. This encounter rate will vary depending on the activity levels and detectability of the animal and trapping effort, and consequently, these estimated index values may not be linearly related to true abundance (Stephens *et al*., 2015). This leads to considerable controversy over their use (e.g. Hayward *et al*., 2015).

Although it has been argued that index sampling is sufficient for the majority of research (Caughley, 1977, p 12), an estimate of relative abundance may not always be suitable for the purposes of a study. Indices can be inconsistent when compared between populations or applied over large geographical areas. There are also inherently high levels of error an uncertainty involved in these estimates which are often overlooked, unless the data are standardised to estimate absolute abundance (Stephens *et al*., 2006). Consequently, absolute population numbers can allow many conservation and management tasks to be conducted with greater confidence, such as understanding predator-prey relationships, which requires comparing data from multiple species (Baker *et al*., 2006), or setting hunting quotas .

The use of relative abundance values for studying rare species, or those that occur in low densities, is highly controversial (Hayward and Marlow, 2014). Differences of just a few individuals between estimates of relative abundance and the true abundance can dramatically alter conclusions about the state of the population. For example, population estimates of brush-tailed rock wallabies obtained using faecal counts suggested that a small colony remained in the Grampians, Victoria, but were unable to detect that the population had been reduced to a single individual (Reside, Victorian Brush-Tailed Rock-Wallaby Recovery Group, unpubl, cited in Piggott and Taylor, 2003).

More fundamental problems with index sampling have also been suggested. Marks *et al*. (2009) note that many of the factors measured in index surveys (e.g. bait uptake rate, scat deposition rate, rate of detection by spotlighting) may be affected by behavioural changes rather than changes in abundance. This can lead to misleading data if changes in relative abundance estimates are assumed to correlate to proportional changes in abundance. Anderson (2001) goes further, and suggests that the fundamental assumption that count data relate closely to population size or density is critically flawed. He argues that for indices of abundance to be useable surrogates for true abundance, the relationship between the two (determined by the probability of detection) must be constant across different observers, environments and populations. However, this is often not the case (see also Kohn *et al.,* 1999). Anderson (2001) also notes that many of the factors which can influence the detectability of a subject, and affect the count, exhibit temporal trends; even for surveys of a population in a single location, day-to-day variation in observer vigilance, subject behaviour and weather may alter the probability of detection. Unless this temporal variation in

detectability is independent of abundance and of much lower magnitude than the variation in the population size that is the subject of a study, then it can render such indices unreliable.

Index surveys are can use data collected for other purposes, or collate data collected using different methods to make inferences about populations, and can often be used in circumstances that severely limit the capacity for producing reliable census or mark-recapture population estimates. Measuring true abundance, though preferable to the use of indices, can be difficult or impractical. Counting every individual in a population is an extremely time-consuming task (Marks *et al*., 2009) and is impractical for cryptic or elusive species, or large study areas. Mark-recapture methods can be prohibitively expensive for extensive use (Davey *et al*., 2010). For wider-scale studies, nation- or species-wide censuses are impossible for the majority of species (Davey *et al.,* 2010).

For many studies, then, index sampling may be a more practical method of gauging population size, especially for large scale studies. Statistical methods such as generalised linear models (GLMs), or poisson distributions can be useful when handling data from counts of rare individuals that do not fit conventional distributions, and can be used to produce species distribution models from index counts (Guisan and Zimmermann, 2000). Long-term population response surveys (e.g. Jacquot *et al.,* 2013) may also be able to measure changes in population size using measures of relative abundance, without requiring data on the absolute abundance. However for many tasks, sampling methods that produce an estimate of actual, rather than relative, abundance will still be substantially preferred. At the very least, such studies would provide useful data for the calibration of index sampling data, in order to determine the circumstances in which they may be useful (or otherwise) (Loison *et al.,*2006).

As an alternative to index sampling, population estimates can be produced using mark-recapture studies. These still produce estimates of abundance that are not relative to trapping effort, but are less time-intensive than census methods like those used in Baker *et al.* (2006). This makes them more practical for studies of larger areas or over longer timeframes, and for species that are elusive, or otherwise difficult to count.

Traditional mark-recapture methods involve the physical capture and tagging of individuals. As such, they are considered invasive, as they require extensive and potentially repeated handling of individuals. In this context, the term *invasive* (as discussed by Backay *et al.*, 2008) refers to methods that require direct handling or observation of target animals by the researcher, and does not imply inherent judgement of the methods. Indeed, invasive capture methods allow the collection of

specific biological information such as accurate weight measurements (e.g. Hoyle *et al.*, 1995), and tagging captured individuals with telemetry devices can provide detailed information on spatial behaviours such as territoriality and ranging behaviour that are unavailable using non-invasive methods (Johnson *et al*., 2013). These spatial data can be extremely important for monitoring wildlife management schemes, and species responses to them (Johnson *et al*., 2013).

The downside to traditional mark recapture methods is that the necessity for direct contact with target species can create feedback within the system being studied, which may be difficult to predict (Piggott and Taylor, 2003). For example, lasting negative effects on captured northern hairy-nosed wombats (*Lasiorus krefftii*), resulted in recaptures weighing significantly less than at first capture, and with significant weight loss still observed in individuals recaptured between 30 days and 6 months after initial capture (Hoyle *et al*., 1995). Furthermore, when areas were trapped again after a 3 week gap, population estimates from the second session were lower, suggesting a dispersal response to the trapping. In addition to negative condition effects being ethically and ecologically undesirable (especially for studies of vulnerable or endangered populations), the evidence of dispersal responses suggests that invasive mark-recapture studies could be unsuitable for making accurate population estimates of some animals. It is possible to mitigate some of the negative feedback effects of physical capture through careful study design, for example by using trappability estimates to account for variation in trap responses within the population studied (Krebs and Boonstra, 1984), or statistical methods to account for changes in behaviour following trapping (Otis *et al*., 1978). By and large, however, population estimation studies are increasingly switching to less invasive study methods.

There are a couple of useful alternatives to traditional mark-recapture methods in this regard. Remote camera traps can be used to perform non-invasive population estimates of some species on a similar principle, by substituting the use of tags for recognition of individuals from photos. However, this requires the target animals to be readily visually distinguished, and can thus be unsuitable for the majority of species. A second alternative is the use of genetic analysis to enable the identification of individuals from remotely collected samples (Kendall and McKelvey, 2008). This involves the collection of hair or scat samples from the target animal, and the extraction and analysis of DNA from those samples. Non-invasive genetic sampling (NGS) enables confident differentiation of individuals once protocols have been developed, without requiring the researcher to come into contact with the subject animal (Sheehy *et al.*, 2014), making it especially advantageous for studying visually or behaviourally cryptic species (Nuske *et al.*, 2014). Several studies have also demonstrated an improvement in the precision of population estimates produced using NGS over traditional mark-

recapture methods (Waits and Paetkau, 2005), including a study by Banks *et al*. (2003), which provided an improved estimate of the *L.krefftii* population referred to above.

In comparison with methods such as camera trapping, or tagging studies that gather subsequent resight data, non-invasive genetic sampling initially provides limited biological and spatial information (Sheehy *et al.,* 2014). However, the initial data are no more limited than for many capture-mark-recapture studies, and the use of sex-specific primers in the analysis can allow the sex of sampled animals to be determined (e.g. Berry *et al.*, 2012) and the genetic data collected can allow a range of further population analyses to be conducted (Piggott and Taylor, 2003). NGS has been found to be more cost effective than telemetry tagging (Johnson *et al.*, 2013), and has a lower start-up cost than camera trapping, without the risk of theft that the latter carries (Weaver *et* al., 2005). Remote sample collection is also more time efficient than abundance counts and traditional mark-recapture, the latter of which can take months or even years to build up a suitable sample size, during which time the population size could change significantly (Piggott and Taylor, 2003).

There are some limitations to remote genetic sampling. Waits and Paetkau (2005) note that non-invasive mark-recapture studies often suffer from low sample sizes in comparison to camera-trapping, due to the need for target organisms to come into contact with the trap. This is compounded by the possibility that collected hairs might not be successfully amplified and analysed (Waits and Paetkau, 2005; Piggott and Taylor, 2003), although some studies (e.g. Sloane *et al.*, 2000) report extremely low error rates, suggesting that the use of methodological precautions can significantly reduce the risk of errors. Furthermore, genotyping errors can lead to a 'shadow effect' when the method is used for mark-recapture studies whereby newly-captured individuals are incorrectly identified as recaptures, which is unique to the genetic method (Mills *et al*., 2000). Because these methods are still relatively novel, studies can often require significant research and development to isolate suitable methods for the study population, and to minimise the risk of such errors, which can be costly in terms of both time and resources. However, once a successful protocol has been established, it can be used repeatedly in future studies.

Despite these issues, non-invasive genetic sampling is increasingly relied upon to produce population estimates for a variety of mammalian taxa (Augustine *et al*., 2014), including a range of ursids, canids, felids and mustelids (Kendall and McKelvey, 2008), as well some marsupials (e.g. Nuske *et al.*, 2014; Banks *et al*, 2003), primates (Amendola-Pimenta *et al.,* 2009), and even forest elephants (Hedges *et al*. 2013). Being neither invasive nor index-based, it offers distinct advantages over many traditional methods of estimating abundance. These include increased capture probabilities, reduced effects of disturbance and no risk of tag loss compared with traditional mark-

recapture (Mills *et al.,* 2000), along with the potential to provide accurate and robust abundance data (Marks *et al.,* 2009; Piggott *et al.*, 2008). There is also the potential for non-invasively collected DNA to be used as an alternative to invasively collected blood and tissue samples for studying population genetics, phylogenetics and relatedness (Piggott and Taylor, 2003), allowing for more detailed study of target populations.

NGS has considerable potential as a tool for studying mammalian ecology, especially for filling the gaps in current knowledge about the abundance of important mammal species, particularly those of management concern. One such example of a mammal of management concern, is that of the red fox (*Vulpes vulpes*). Globally, the red fox is extremely widespread, having the largest distribution of any terrestrial non-human mammal (Schipper *et al.*, 2008; MacDonald and Reynolds, 2008). Native to the Northern Hemisphere, where it occurs commonly across Europe, North America and Russia, it has also been introduced to Australasia, where it is highly invasive, and poses a threat to many native and domestic vertebrate species (Vine *et al*., 2009). In Europe, it is associated with the transmission of diseases such as rabies and echinococcosis (Baker and Harris, 2006) and predation of livestock (Moberly *et al*., 2003), game species (Reynolds *et al*., 2010;Baker *et al.,* 2006) and species of conservation concern (Reynolds *et al*., 2010). Consequently, management of red fox populations is widely practised (Newsome *et al.*, 2014; Berry *et al*., 2012, Reynolds *et al*., 2010).

Attempts to control fox populations with isolated shooting programmes (common practice in the UK) may have little or no effect on fox population density (Baker and Harris 2006). Meanwhile, Jaquot *et al*. (2013) observed a significant decrease in fox density in response to a long-term rodent control programme, and Trewby (2008) and Letnic (2012) demonstrated an increase in fox population size in response to badger and dingo culling, respectively. Accurate population data on foxes is therefore of great importance in order to assess the need for fox control, and to determine how fox population size changes in response to control measures.

Studies of population size in European, and especially British, *V. vulpes* populations have been almost exclusively limited to the use of index sampling methods such as spotlight counts (e.g. Baker and Harris, 2006) and faecal counts (e.g. Webbon *et al*., 2004). National estimates are often generated from surveys of numbers of animals killed (Davey *et al.*, 2010), or more recently from a media-driven survey of fox sightings by UK residents (Scott *et al*., 2014). Whilst these surveys may be useful for the detection of long-term population trends, the potential pitfalls of index surveys already discussed mean that there is a need for accurate abundance data to calibrate the existing

data and improve our understanding of fox abundance and its relationship to relative abundance. Non-invasive genetic sampling has the potential to be a useful tool to achieve this.

The overall aim of the study was to determine the efficacy and feasibility of using these methods to produce population estimates by applying capture-mark-recapture principles to non-invasively collected samples, for UK red fox populations. NGS can be applied to either scat or hair samples, but genetic analysis of scat may run the risk of contamination by prey DNA found in the scat. Both scat and hairs may be tricky to obtain where foliage is dense, or foxes are at low density, but this can be mitigated by using lures to draw foxes to the target location. To this end, several non-invasive trapping methods were tested for their usefulness in collecting hair samples red foxes in woodland in Durham, as a less labour-intensive approach than scat detection. Different lures were used, with the aim of determining whether a scent lure or meat bait were more successful at attracting foxes to a trap, and traps were set up with different hair collection structures in order to determine which were most successful at collecting hairs from animals which visited the traps. Laboratory protocols for extracting and analysing DNA from fox hairs were also trialled, to determine a method for identifying individual red foxes from these samples.

Chapter 2: Non-invasive traps for collecting Red Fox hair

Introduction

Non-invasive genetic sampling (NGS) has already been successfully used in Australia, to study the effectiveness of poison baiting as a control for *V. vulpes* (e.g. Berry *et al.*, 2014; Berry *et al.*, 2012; Marks *et al*., 2009), and the method is becoming widely used to study invasive fox populations. However, attempts to use NGS to study foxes in Europe have been limited. Monterroso *et al*. (2014) performed a pilot study to compare camera trapping with genetic sampling for detecting the presence of several mesocarnivores, and concluded that camera traps were more suitable for that purpose due to a combination of low target density, and low detection rates when using genetic sampling. A similar study in Australia by Vine *et al*. (2009) seems to confirm the problems with NGS at low fox density. However, a confounding problem is that the sample collection methods used in these studies differ both from each other, and from those used in successful studies by Berry *et al*. (2014) and Marks *et al*. (2009).

There is no clear consensus on how best to collect samples for the study of red foxes. Whilst the majority of NGS surveys use traps designed to collect hair from the target animals, some (e.g. Marks *et al*., 2009) use DNA extracted from faecal samples. Vine *et al.* (2009) found that faecal samples provided higher detection rates than hair samples, although for genetic analysis, faecal samples have often been found to contain less usable DNA, and higher levels of contamination than hair samples (Amendola-Pimenta *et al.,* 2009; Waits and Paetkau, 2005). Berry *et al.* (2007) suggested a method for performing species-specific PCR has that could reduce the impact of contamination on the amplification of faecal DNA, but its effectiveness is currently disputed (Goncalves *et al*., 2014; Sarre *et al.*, 2014), and faecal sampling remains the less popular means of collecting non-invasive genetic samples.

Even in studies that only collect hair samples, no clear consensus has yet emerged on the best trapping method. Hair traps consist of a combination of bait and hair collection surfaces, arranged so that in investigating the bait, the fox will deposit hair on the collection surface. Baits used vary, and include both scent lures and food baits. Of the scent lures, some are designed to elicit a rubbing response from territorial animals (e.g. Monterroso *et al*., 2014), whilst others are food-based to invite investigation (Vine *et al.*, 2009). Hair collection structures also vary, and include barbed wire (Kendall and McKelvey, 2008), brush pads (Monterroso *et al*., 2014) and double sided tape (Berry *et al.,* 2014). Whilst there are studies that compare subject responses to different types of bait or lure (e.g. Moseby *et al*., 2004; Hunt *et al.*, 2007; Saunders and Harris, 2000), they can

suffer from low sample sizes (e.g. Moseby *et al*., 2004; Hunt *et al.*, 2007) and are few and far between.

The wide variation in methodology, combined with the extensive geographic range of the target species, and comparative lack of studies, results in a need for pilot studies to determine the efficacy and feasibility of NGS for estimating red fox abundance in the UK and Europe, before it can be widely used as a management tool. Furthermore, because remote genetic sampling is still a relatively new tool, there have been few studies that have compared the effectiveness of different methods. As such, and potential NGS studies of UK foxes are likely to require significant investment of both time and resources into researching and developing appropriate data collection methods, something which is likely to reduce the attractiveness of these methods.

To this end, this study trialled several different methods of hair collection, to determine their usefulness for monitoring red fox populations in woodland in the city of Durham. In part, this information could be used to determine whether and how to proceed with further monitoring of red foxes in the area. Four different trap types were trialled, incorporating different combinations of hair collection structure (using either barbed wire or double-sided tape as a hair-collection surface) and bait (using either valerian oils to elicit rubbing behaviour, or food baits).

Methods

Study area

The study area (figure 1) encompassed two adjacent woodland sites in County Durham: Great High Wood and Little High Wood. The sites are in very close proximity to each other, and it was suspected that they may both be used by the same fox population. The sites are owned by Durham University, and are located to the South of Durham city. Little High Wood is bordered by University buildings to the North and South, whilst Great High Wood is bordered by stocked pastoral land to the South and East, and University buildings to the North and West. Both are deciduous woodland, characterised by oak, beech and sycamore trees, which provide near full canopy cover in summer. Both sites are on sloping ground with little to no standing water throughout the year, and receive an average of 643mm rainfall annually.

Figure 1: An aerial map of the study area, labelling Little High Wood (blue), and Great High Wood (orange). Durham University Department of Biological and biomedical sciences lies between the two wooded areas.

Both sites have several footpaths running through them which are used by runners and walkers, often accompanied by domestic dogs. Great High Wood is also occasionally visited by cyclists and horse riders. The area was being monitored using camera traps both before and during this project as part of ongoing Durham University research, and some cameras were used to observe the hair traps on an ad hoc basis. Red foxes have been extensively documented in both woodland areas but have not been subject to control nor does there appear to have been any documentation of spatial use by the species in the area. Thus, identifying the efficacy of the hair traps would not have been limited by the presence of the focal species.

Trapping

Three distinct types of hair collection structure were tested. The first, a 'sticky wicket' snare, was similar to the design used successfully by Berry *et al*. (2012) to collect red fox populations in Australia. It consisted of three wooden posts (approx. 50cm tall), which were wrapped first in a layer of gaffer tape, followed by a layer of double sided tape (Wickes double sided flooring tape). The posts were driven into the ground approximately 7cm apart at the base so that they were approximately 12cm apart at the top. These structures were positioned at the entrance of u-shaped enclosures constructed from locally collected wood and plant debris to create a narrow, closed-off corridor approximately 30cm wide and 100cm long (Figure 2). The structures were baited with meat at the far end of the corridor from the 'wicket', such that hairs would be collected from animals as they brushed past the sticky posts in order to investigate the bait. Meats used included processed turkey, raw chicken, tuna, and rabbit, rat and squirrel carcasses. Animal carcasses were provided by staff of the Durham Botanical Gardens, and had been culled as part of ongoing pest management. Other meats were purchased locally. Eight traps were placed between December and April, for a total of 200 trap nights.

Figure 2: Sticky wicket trap

The second trap design used meat baits, which were affixed to the top of a fence post (approximately 100cm tall) using a barbed wire staple. The posts were wrapped in barbed wire, and driven firmly into the ground so that they remained upright, with the bait held off the ground (Figure 3). This design was intended to force animals investigating the bait to climb the post, snagging hair

on the barbed wire in the process, and was described by Kendall and McKelvey (2008) as an effective method for collecting hair from foxes. The baits used were the same as for the sticky wicket traps, and baits on both these traps and the sticky wickets were replaced either when they had been consumed or after five days. Eight traps were placed for a total of 148 trap nights.

Figure 3: Meat-baited wire-wrapped post trap

The third trap was based on methods used by Monterroso *et al.* (2014), and was designed to exploit canid neck-rubbing behaviours. It consisted of a single 50cm wooden post, covered with a hair-collection surface, and baited with a scent lure, consisting of small strips of cotton gauze, soaked in approximately 5ml of valerian oil, which has been said to be effective in attracting canids such as foxes (Monterroso *et al*., 2011; Velli *et al*., 2015). The cotton strips were deployed inside perforated plastic tubes, which were attached to the outside of the post using tacks and spaced 15cm apart. These traps were deployed with two different hair collection structures: one set was deployed with a layer of double-sided tape over a layer of gaffer tape, similar to the posts in the sticky wicket traps (Figure 4); the second set was wrapped in barbed wire. They were set up such that an animal rubbing up against the post would be likely to snag some hairs on the tape or wire. Eight barbed wire posts were set up and monitored for a total of 176 successful trap nights, and six

sticky tape posts were set up and monitored for a total of 189 successful trap nights. Scent lures were replenished every seven days.

Figure 4: Scent-baited sticky post

Trapping took place between November 2014 and July 2015. The traps were deployed randomly across the survey area, avoiding locations with dense undergrowth to ensure that the snares were accessible, and avoiding high proximity to paths used by dog walkers to limit excessive interference from domestic dogs. When each trap was deployed, the plant cover at ground level and understorey level was estimated for 2m in all directions (to the nearest 10% cover of that area), to gain an estimate of the accessibility of the trap to mammals and birds. The tape on double-sided tape traps was replaced when it became wet or was no longer sticky. Some traps were vandalised or removed during the study period, and trap nights during which these incidents occurred were discounted from the totals.

Hair collection and identification

Traps were checked daily when possible, with no more than three days passing between checks. Hair collection surfaces were checked thoroughly, and any hairs were removed using

tweezers and stored in plastic bags in freezers at -20°C until lab processing. Hair samples were examined first by eye and then under a microscope, species designations were determined with the aid of a hair identification guide (Teerink, 2004), and by comparison to known fox samples. Where possible, results were checked using data from camera traps. Samples that could not be positively identified were listed as unknown. Unknown samples that could not be readily distinguished from fox hairs would have been considered as potential fox samples, and compared to known fox samples following genetic analysis.

Data analysis

A generalised linear modelling approach was used to identify the importance of various factors in predicting the likelihood of trap visitation and hair deposition. Factors considered were trap design, bait type, trap location, understorey plant cover, shrub-level plant cover, time of year, moon phase, and minimum overnight temperature (table 1). Temperature data were obtained from the records of Durham University Observatory. A day^2 term was also included, to investigate the possibility of a diminishing effect of time of year over time, to account for the potential of reduced fox activity over winter and towards the end of the year.

Table 1: table of predictors

Predictor	Metric	Value range	Source
Trap design	none (categoric)	Sticky Wicket, Bait post, Wire Post	NA
Bait type	none (categoric)	Meat, Scent	NA
Trap location	none (categoric)	NA	NA
Understorey cover	%	0-100	Measured on site
Shrub cover	%	0-100	Measured on site
Time of year	Julian day	1-365	NA
Moon phase	none (categoric)	full moon, last quarter, new moon, first quarter	www.timeanddate.com
Minimum overnight temperature	°C	(-2.1) to 13.3	Durham University Observatory

To investigate these factors, a set of binary logistic regression models was generated to explain variation in hair capture success using all possible combinations of factors, using the 'glm' function of the 'ggplot2' add-on for R. These were then dredged using the 'dredge' function in the same program, and ranked according to their delta-AIC values; models with delta-AIC values greater than 6 were excluded as having too little support from the data (Richards, 2008). To prevent the retention of overly complex models, model were excluded if a simpler version (including fewer

factors, with no additional factors) existed with a lower delta-AIC value (Richards, 2008). Models were also excluded if they included the factor 'day^2 ' but excluded 'day', as the two are clearly linked, but the former will have a higher chance of detecting the effect. The remaining models were ranked according to their Akaike weight (as described by Richards *et al*., 2011), which were used to estimate the probability that each model was the most parsimonious. Parameters were weighted according to the sum of the weights of the models which incorporated them, in order to determine which were the most useful explanatory factors.

Results

Table 2 details the number of trap nights, hair collection events and bait take events for each trap type. Of 65 hair samples collected, 64 were collected on sticky wicket traps. Of these, 0 were identified as being from foxes. 47 (73.4%) were identified as badger (*Meles meles*) samples, 8 (12.5%) were from dogs, 3 (4.7%) were from rats and 6 (9.4%) were unknown samples. All of the unknown samples were sufficiently distinct from fox samples to be discounted from further analysis. One hair sample was collected from a meat post trap, and was identified as being from a badger.

Table 2: Summary of the total number of trap nights, number of trap nights when bait was taken, and number of trap nights when hair was collected, by trap type

trap type	bait type	Trap nights	bait taken	hair collected
Sticky post	Scent	189	0	0
Sticky wicket	meat	200	127	64
Bait post	meat	151	135	1
Bait post	Scent	176	0	0

Analysis

Due to the lack of fox samples collected, hairs collected from all species were included in the analysis. Model selection identified support for six models (table 3), and the parameters were weighted according to the weight of the models that included them. (Figure 5).

Table 3: set of most parsimonious hair collection models. The following variables were not included in any of the most parsimonious models: minimum temperature, shrub cover, location, and understorey cover.

(Int)	bait type	day	day^2	moon phase	trap type	K	AICc	delta	weight
-5.91E+01		2.50E-01	-2.83E-04	+	+	4	260.1	0	0.429
-8.45E+00	+	9.24E-03		+	+	4	262	1.9	0.166
-3.92E+00	+			+	+	3	262.7	2.6	0.117
-9.58E+00		8.52E-03		+	+	3	262.9	2.8	0.106
-1.09E+02		4.75E-01	-5.38E-04		+	3	263.2	3.1	0.091
-5.21E+00				+	+	2	263.2	3.1	0.091

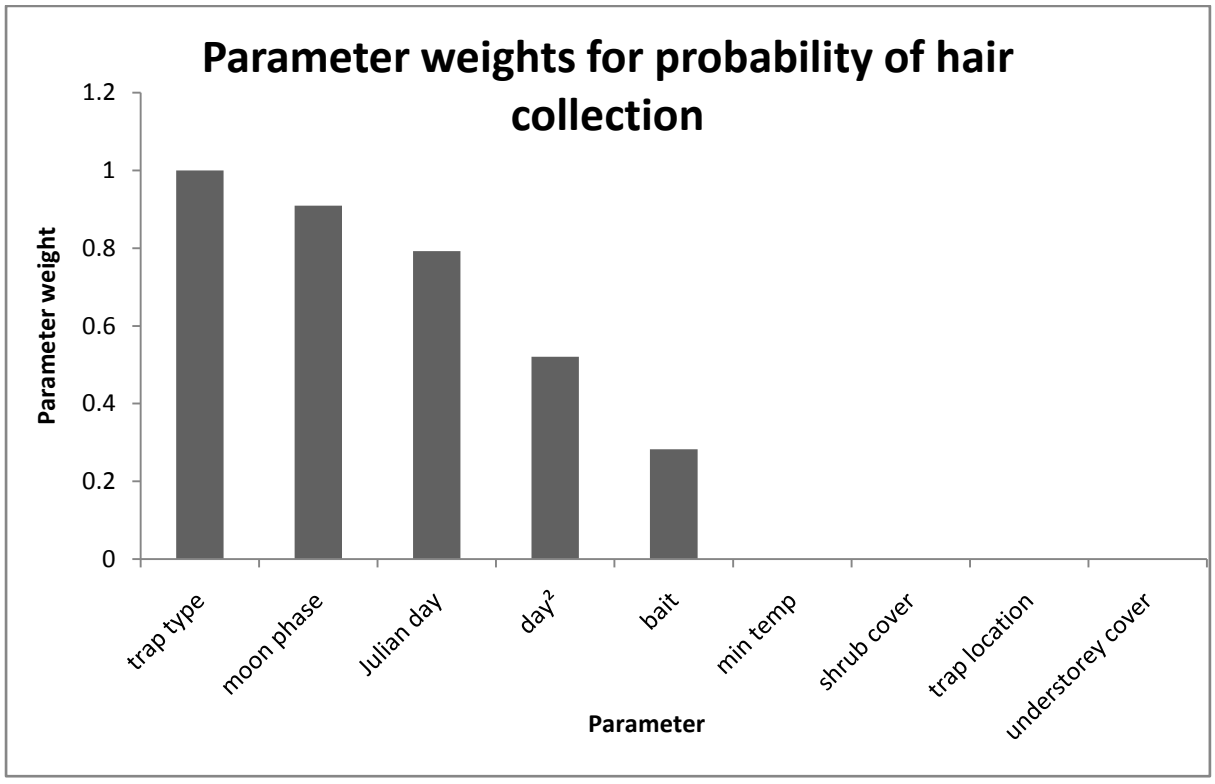

Figure 5: Parameter weights for factors affecting the probability of hair collection

Trap type was the most highly weighted parameter, and was found in all of the final models. Temporal variation was also an important explanatory parameter, with both moon phase and Julian day weighted highly. The highest chance of hair collection occurred in spring, and hair collection probability is highest during the new moon and first quarter moon phases (Fig. 6). Day^2 was also weighted highly, suggesting a drop-off in trap success towards the end of the year.

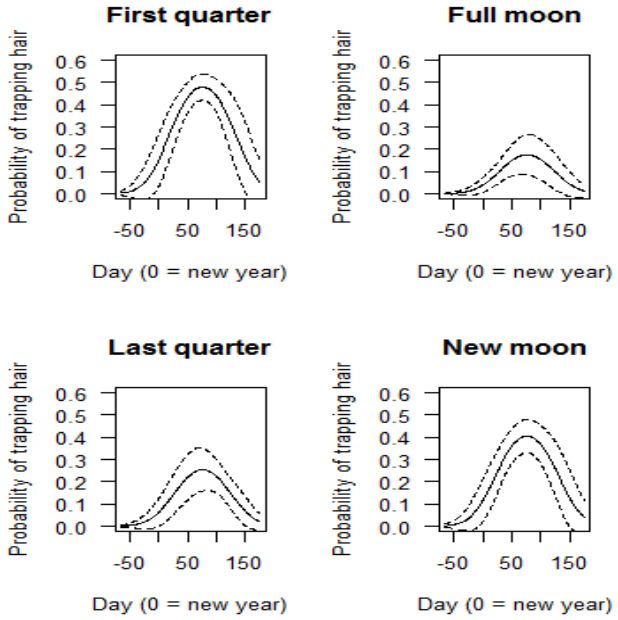

Figure 6: relationships between moon phase and Julian day, and the probability of trapping hair for the sticky wicket trap, generated using the model with the highest explanatory weight. The dotted lines represent standard error.

Discussion

Three trap designs were tested, which incorporated two different types of bait and two different forms of hair collection structure. Although no fox hairs were collected during the study, the traps were visited by a range of animals, and some designs did collect hairs, with the sticky wicket being significantly more successful than other designs. This information will be discussed in the context of two main issues: trap success; and implications for monitoring fox abundance in Durham City and beyond.

Trap success

Despite the documented presence of red foxes at the study sites, all of the trap types used in this study were unsuccessful in collecting fox hairs, which is in stark contrast to the results of other surveys which used similar traps (e.g. Berry *et al*., 2012; Marlow *et al.,* 2015; Monterroso *et al.,*

2014), all of which were able to collect fox hairs. This lack of success could be attributable to a variety of factors.

Firstly, although foxes had been seen on the study site prior to beginning this study, the site was relatively small in comparison with those used in other hair trapping studies, and the fox population in the area may therefore have been relatively small, especially if the study site were to contain a stand-alone population. This is unlikely to be the case, however, as the area contains many fragmented patches of woodland, open fields, and quiet roads (Figure 1), none of which are likely to cause a significant barrier to fox movement.

Secondly, there may have been an adverse effect of competition from other species; the results from the sticky wicket hair traps indicate an active badger population, and an unpublished study by Trewby (2008) indicates that badgers can outcompete foxes when their ranges overlap. This may have led to a reduction of fox population density in the study area, and potentially could have deterred foxes from feeding from traps at which badgers regularly fed. There were two instances from camera trap data of foxes approaching traps, but leaving without interacting with them, which implies that they were wary. However, this may have been related to a fear of humans rather than badgers, since the traps were checked very regularly and would likely have retained some human scent.

In retrospect, the placement of the traps may also have been a significant factor. In order to avoid interference by humans and domestic dogs, traps were placed away from paths. However, this may have exacerbated the issue of badger interference, as badgers tend to frequent areas with more undergrowth. Furthermore, as foxes often follow existing trails, the traps may have received higher rates of fox visitation closer to paths, which is definitely worth considering for future studies. I

Finally, evidence from camera traps showed interference by corvids at traps baited with meat. Corvids often took bait very soon after it the traps were set. This was most prominent in the meat post traps, possibly due to the bait being raised off the ground and more visible from the air. By taking the bait so early, these birds may have prevented other animals from engaging with the traps, which fits the high bait-taken and low hair-collection rate observed for these traps.

Despite the lack of fox samples, the decision was made to continue trapping, to test for the presence of any seasonal changes in behaviour that could affect visitation rates. Although many more badger hairs were collected, the focus remained on the effectiveness of the methods for detecting foxes, as one of the aims of the project was to enable future fox monitoring projects in the area. Nevertheless, the presence of collected badger hairs allows some inferences to be made about

the effectiveness of the hair collection designs. No hairs were collected by either trap type that was baited with scent, and only one sample was collected on a meat post trap. This suggests that double-sided tape used in a sticky wicket trap design was a more effective hair collection surface than barbed wire post traps. A useful focus for future studies could be to attempt to dissociate the trap design from the collection structure, by using wire-based sticky wicket traps.

Of the two bait types used, the meat baits were the only ones to elicit any response from wildlife. The lack of response to the valerian scent lures was unexpected, and differed from the results of previous studies (Monterroso *et al.*, 2011; 2014). This lack of success may have been due in part to the regular rainfall during the study, since scent lures can become less effective, and require more regular refreshing, when exposed to rain and snow (Schlexer, 2008). The rainfall may also have impacted on the usefulness of some of the hair collection structures. Double sided tape was often found to be less sticky and to need replacing following overnight rainfall, which could potentially have prevented samples from being properly collected on these occasions, and reduced trap success.

Implications for monitoring fox abundance

The lack of success of the traps tested in this study for collecting fox hairs means that, at the very least, further testing would be required to determine a suitable non-invasive hair collection method for studying this population. The success of the traps used in this study was much lower than in other studies that used the same or similar designs, which suggests that there could be some additional challenges associated with the use of these traps in UK woodland environments. Complex environments such as dense woodland have been reported to inhibit the dispersion of odours (Leigh and Dominick, 2015), which could reduce the effective range of scent based lures such as valerian oil. Along with the UK's high levels of rainfall, this could reduce the usefulness of such lures in UK woodland studies.

The sticky wicket traps showed the most potential in this study, and were able to collect hair from a range of animals. However, whilst camera footage showed that some foxes were drawn to investigate these traps, they appeared to be too cautious to enter. It is possible that these traps could be used to monitor UK red foxes with some modification. Gustatory additives such as beef stock have been shown to enhance the attractiveness of food bait to captive red foxes (Saunders and Harris, 2000), and may improve visitation rates. The use of other scents such as fox urine to cover any human scent may also be usefully investigated in future trials.

It would also be useful to find out more about the nature of the fox population in Durham City Woodland, to understand more about their habitat use, and ensure more effective trap placement. As the fox population in the area has not been extensively studied, it is uncertain whether they are part of an urban or rural group; although the site is close to Durham City centre, and bordered by buildings to the North, the area to the South is predominantly farmland. It is also not certain whether the area contains a fixed population, or a transient one, which could have important implications for understanding their habitat use. Transient populations in particular can be hard to study, as they range over large areas (Dekker *et al.*, 2001), so the trapping effort required would vary greatly depending on these behaviours. Further study of the population could increase the effectiveness of future trapping efforts.

If a reliable method of collecting hairs is not found, another potential focus for future studies is to investigate the use of scat samples NGS studies of UK fox populations. Scats could be a viable alternative to hair samples, as they can be analysed using the majority of the same methods. Scat collection trials using different forms of lure could investigate whether samples can be reliably collected in sufficient numbers for use in these studies, and assess the need for, and cost of, trained scat-detection dogs.

In conclusion, despite the challenges raised by this study, non-invasive genetic sampling remains a useful tool for monitoring fox populations. However, if remote hair collection and analysis is to be used to study foxes in Durham City, and other UK woodlands, more trials are required in order to determine a bait that successfully attracts foxes in sufficient numbers for abundance estimates. Otherwise, scat collection should be investigated as an alternative NGS tool.

Chapter 3: Laboratory methods for analysing red fox DNA from hair samples

Introduction

Non invasive genetic sampling can provide DNA samples which can be used as an alternative to blood or tissue samples for a variety of studies (Piggott and Taylor, 2003). Simple genetic analyses can be conducted on samples to enable the species of the sample to be identified. This can be used to confirm the presence of a rare or elusive species from signs such as scat or hair (Monterroso *et al*., 2014). It is also often used in conjunction with other genetic analyses to identify and remove samples from non-target species, prior to performing a full analysis of samples (e.g. Velli *et al.,* 2015). However, the hairs of many species can be distinguished by careful visual analysis (Teerink *et al.*, 2004), so this approach is most useful for distinguishing closely-related species with similar hair structure. An alternate approach involving species-specific PCR protocols, which only amplify DNA of target species, has been used in some studies (e.g. Berry *et al.*, 2007). However, these methods are still relatively new, and Gonçalves *et al.* (2014) have reported that one such method did not successfully exclude the DNA of non-target species from PCR, making it risky to rely on this technique alone when excluding false samples.

Individual identification can also be performed using non-invasively collected genetic samples by comparing the lengths of a series of variable genetic markers. DNA microsatellites are very useful for this purpose, as their short, repeating sequences are prone to copy errors. These errors produce different alleles at the locus, with different numbers of the repeating sequence, and so different lengths. The combination of the lengths of a specific set of microsatellite loci can be measured to produce a genetic 'fingerprint'; samples from different individuals can be distinguished by differences in the lengths of the sequences at one or more of these loci; multiple samples from the same individual will have identical 'fingerprints'.

This technique is used to produce accurate estimates of the number of individuals in a population, by applying the principles of capture-mark-recapture studies to non-invasive surveys. These surveys can also detect trends in the size of a population, or even the movement of individuals between populations, and has been successfully used to monitor the recovery of red fox populations following control schemes (Berry *et al*., 2014). Furthermore, as the individual tags are linked to genetic variation, the same methods can be used to study additional population dynamics, such as historical dispersal patterns, by comparing genetic distance between individuals and populations with their geographical locations (Stanton *et al*., 2015).

This method has limitations; microsatellite analysis for the purpose of individual recognition lacks a procedure for differentiating species, and so requires either an additional genetic analysis step or detailed analysis of the samples to ensure that only samples of the target species are included, which can be costly and time-consuming when using samples of unknown provenance. Furthermore, remotely collected hair samples often yield very low quantities of DNA, which can lead to unsuccessful analysis of samples, especially in the extraction method is not very efficient (Piggott, and Taylor, 2003). Finally, to be able to reliably distinguish between individuals, the set of primers used must be suitable for the target population; if they are not, sufficiently variable, there is a risk of falsely identifying two samples as the same individual (Mills *et al*., 2000). If the population has not been studied before, a range of primers may need to be tested to find a set that can be used for analysis.

The aim of this study was to identify a protocol for the genetic analysis of samples of non-invasively collected red fox hair. This involved identifying and testing a suitable method for DNA extraction, which would be able to reliably extract DNA from samples as small as a single hair; hair collection traps can yield low numbers of plucked hairs from a single visit, and by extracting DNA from single hairs, it is possible to be sure that hairs from multiple individuals are not included in the same sample. Although extraction kits are often used for this purpose, chelex resin is also commonly used and, in addition to being relatively inexpensive, requires only one step to extract DNA, reducing the risk of contamination of loss of genetic material during the extraction process (Piggott and Taylor, 2003).

Microsatellite markers have been identified for studying red fox populations in Australia (Berry *et al*., 2012; Marlow *et al*., 2015) and Europe (Mullins *et al*., 2014). However, although these populations are closely phylogenetically linked to UK foxes (Statham *et al*., 2014), the three are geographically isolated, and markers established for one population may be less useful for studying another. Markers selected from these studies were tested on UK fox samples, to determine a set that could be reliably used to identify individuals.

Additionally, DNA was extracted from hairs collected non-invasively from domestic dogs (*Canis lupus familiaris*), which is the most closely-related UK species to the red fox, and the most likely for hairs to be mis-identified as foxes. Furthermore, the microsatellite markers that were tested had all originally been designed from dog sequences, meaning that it was likely that dog samples would amplify successfully at these loci using the same primers. The dog samples were therefore analysed and compared with fox samples, in order to identify whether such accidental

contamination would be identifiable at a genetic level, and determine the utility of genetic species-identification techniques in UK fox studies.

Methods

Samples

Hair samples were donated from 8 foxes which had been culled as part of ongoing population control by a landowner in Yorkshire. A large number of hairs were plucked from each carcass shortly after culling and hairs from different individuals were stored separately in sealed plastic bags at -20°C. The sex of each individual was recorded prior to removing hairs; there were 2 male and 6 female foxes. These samples were used for primer selection and genetic analysis.

Samples from 4 domestic dogs were retrieved from the sticky wicket hair snares described in the previous chapter, and identified visually by eye and with the aid of an optical microscope and identification key (Teerink, 2004). These were removed from the traps using tweezers and stored in separate sealed plastic bags at -20°C. The dog hairs had not been collected when primer selection occurred but were used for genetic analysis, in order to test how the methods work for closely-related non-target species.

To avoid contamination with human or otherwise non-sample DNA all samples were removed and handled only when wearing gloves, which were changed in between handling different samples, and tweezers and other tools used to handle them were sterilised in 100% ethanol before and afterwards.

Extraction

All extractions were performed using Chelex®, according to the following protocol: 1-2cm of hair was placed follicle-down in 300 µl of 20% Chelex in a tube, and vortexed for 10-15 seconds. The tube was then spun at 10,000rpm for 10-15 seconds in a microcentrifuge to ensure that the sample was in the chelex slurry, before being incubated for 20 minutes at 95°C. Following incubation, samples were again vortexed for 10-15 seconds, and spun at high speed in a microcentrifuge for 10-15 seconds. The supernate was then extracted and stored at -20°C until needed.

To test the effectiveness of the chelex method for extracting DNA from plucked hairs, 56 hairs were taken from the fox and dog samples, and extracted following the described protocol. Drops of 1 µl from each of the resulting extractions were placed one by one on a calibrated nanodrop machine for analysis, and the concentration of DNA in the drop was recorded for each extraction.

Primer selection

An initial list of primers for 16 microsatellite loci and 1 sex-linked locus was collated from two papers that used fox microsatellites for individual identification (Berry *et al*., 2012; Mullins *et al.*, 2014), and is shown in table 4. These loci were amplified separately for each fox DNA sample 3 times with each of the following annealing temperatures: 60°C, 58°C, and 56°C. The optimal annealing temperature for each primer was determined from these samples following electrophoresis as the highest of these temperatures for which at least 6 of the samples were successfully amplified at that locus for all 3 repeats.

All PCRs were conducted using a three primer system, as described by Schuelke (2000). The PCRs used a 25µl reaction mix with 1µl forward and 1µl reverse primer, 1µl of fluorescent marker (FAM, NED or HEX), 1 µl of DNA sample, 0.25 µl MyTaq DNA Polymerase, and 5 µl MyTaq PCR reaction buffer. All PCRs used the following cycling conditions: an initial denaturation at 95°C, for 1 minute, followed by 30 cycles of 95°C for 15 seconds, Annealing temp for 15 seconds, 72°C for 10 seconds, with a final extension at 72°C for 2 minutes. Pre- and post-PCR processing were conducted separately to avoid DNA contamination, and work spaces and equipment were cleansed with ethanol before and after use.

Following PCR, the samples underwent electrophoresis on a 1% agarose gel, which was visually analysed under ultraviolet light using 10x Mydori green dye in order to determine the success of each amplification, indicated by the presence of a fluorescent band on the gel. Microsatellite primers which resulted in a successful amplification of at least 6 of the 8 samples all three times at a single annealing temperature were selected for use in genetic analysis. Based on similar studies, such as that of Berry *et al*. (2014), which had been successful in using microsatellite markers for individual recognition, 8 loci was set as the minimum number that needed to be successful in order to proceed. Four primers were ordered and tested at a time until at least 8 successful primers were found, to minimise cost.

The accuracy of the sex-linked primer (for the SRY locus) was determined by repeating the PCR protocol three times for each sample with the SRY primers and an annealing temperature of 56°C. The products of these PCRs were analysed by electrophoresis as described above. If a band was present on the gel for all three PCR products for a single sample, indicating the presence of the SRY gene (and hence the Y-Chromosome), the test indicated that the sample was male. A sample was classified as female if no SRY bands were present in any of its PCR products. Any samples for which only 1 or 2 products showed bands were discounted, and the process repeated. These results

were then compared with data collected on the sex of the foxes during sample collection, to determine the accuracy of the protocol for determining the sex of an individual.

Table 4: Primers tested for use in genetic analysis.

Locus	Type	Forward	Reverse	Size (bp)	Reference
REN135	Microsatellite	AATTGATTCATGACCCACTAA	GGACCTATTCTGAAGCCTAAC	157-163	Berry *et al.* (2012)
REN195	Microsatellite	GCTTTCCCATTGTGTCCTCA	TGATTGATGCCCTTTCAACA	130-149	Berry *et al.* (2012)
C17.402	Microsatellite	AAATGGGTAATTCATCCAGTGC	CAGGCTTTGTTGAGGTGTCA	80-93	Berry *et al.* (2012)
C27.502	Microsatellite	TTTGAAAGGCTGTATGCATCC	GTTATGGCCAAGTACTCTTCCA	76-78	Berry *et al.* (2012)
AHT142	Microsatellite	AAGCAGATCCTAGAGCAGCA	CCCCACACAGTTTAGAAATATCTGC	132-148	Berry *et al.* (2012)
CXX.374	Microsatellite	GGGTAATTCATCCAGTGCCTT	TATGCAAACATGCAAACATGC	100-112	Berry *et al.* (2012)
C02.466	Microsatellite	TCTGGATTGTGGTCACAACC	ACTGGACACTTCTTTTCAGACG	135-153	Mullins *et al.* (2014)
FH2010	Microsatellite	AAATGGAACAGTTGAGCATGC	CCCCTTACAGCTTCATTTTCC	215-227	Mullins *et al.* (2014)
FH2054	Microsatellite	GCCTTATTCATTGCAGTTAGGG	ATGCTGAGTTTTGAACTTTCCC	143-203	Mullins *et al.* (2014)
SRY	Gene	GAACGCATTCTTGGTGTGGTCTC	GGCCATTTTTCGGCTTCTGTAAG	132	Berry *et al.* (2012)
C01.251	Microsatellite	TACCACTGTCATTTTTCCATGC	AAGAGGATACCGGTGGCAG	128-141	Berry *et al.* (2012)
C25.213	Microsatellite	AATATGGGAGAGGAGAAGAGGG	ATGCTTCCTGGTAAGCAATCA	109-111	Berry *et al.* (2012)
FH2096	Microsatellite	CCGTCTAAGAGCC	GACAAGGTTTCCTG	104	Mullins *et al.* (2014)

Locus	Type	Forward	Reverse	Size (bp)	Reference
		TCCCAG	GTTCCA		
FH2137	Microsatellite	GCAGTCCCATTCCACA	CCCCAAGTTTTGCATCTGTT	185	Mullins *et al*. (2014)
VVM124	Microsatellite	CTCTGCTACACGGCCAAACT	GGTATTCCTGTGCCTCTTGTTC	244	Mullins *et al.* (2014)
VVM189	Microsatellite	GATCTGTGAGCATAAGGGTTTT	TTATCCAGTCCCAAAGTCTGTC	240	Mullins *et al*. (2014)
VVM828	Microsatellite	AGAAGGCACTTGTAAGGTGGAT	GCACACAGACACACATGGAATA	226	Mullins *et al.* (2014)

Genetic analysis

The primers that were successfully amplified in all 3 PCRs at their optimal annealing temperatures were selected for use in genetic analysis using fluorescence capillary sequencing. SRY was not included in this stage of the analysis as it is not a microsatellite gene and so does not vary sufficiently between individuals to be of use in sequencing. The results of these analyses show the sizes of each locus as a coloured peak along a size scale, and different loci that are analysed together can only be distinguished by their size or by the colour of the peak. The primers were therefore arranged into groups according to the size ranges of their corresponding loci (in numbers of base pairs; data on size ranges was obtained from source papers, Mullins *et al.,* 2014, and Berry *et al.*, 2012), such that each group contained no loci with overlapping size ranges. Each of these groups was assigned a different fluorescent marker, so that all loci could be distinguished either by size or by marker. As there were 4 groups and only 3 markers, the loci were then divided into two sets for analysis (groups, sets, and markers are shown in table 5).

Table 5: Results of primer selection process. Loci that amplified at least 6 samples 3 times at the same annealing temperature were selected for use in analysis and are listed as successful. These primers were assigned to fluorescent markers and divided into sets.

Locus	Result of selection process	Annealing Temp. (˚C)	Fluorescent marker	Group	Set
SRY	successful but unsuitable for sequencing	56	NA	NA	NA

Locus	Result of selection process	Annealing Temp. (˚C)	Fluorescent marker	Group	Set
REN135	successful	56	NED	1	2
REN195	successful	56	NED	1	2
C17.402	successful	56	FAM	2	1
C27.502	successful	58	NED	1	2
AHT142	successful	58	FAM	2	1
CXX.374	successful	58	FAM	2	1
C02.466	successful	56	HEX	3	1
FH2010	successful	56	HEX	3	2
FH2054	successful	56	FAM	4	2
C01.251	unsuccessful (did not consistently amplify under trialled PCR conditions)	NA	NA	NA	NA
C25.213	unsuccessful (did not amplify under trialled PCR conditions)	NA	NA	NA	NA
FH2096	unsuccessful (did not amplify under trialled PCR conditions	NA	NA	NA	NA
FH2137	unsuccessful (required number of primers reached)	NA	NA	NA	NA
VVM124	unsuccessful (required number of primers reached)	NA	NA	NA	NA
VVM189	unsuccessful (required number of primers reached)	NA	NA	NA	NA
VVM828	unsuccessful (required number of primers reached)	NA	NA	NA	NA

DNA from each of the samples, including dog samples collected from hair traps, was amplified for each of the microsatellite loci using the PCR conditions described, with the fluorescent markers that had been assigned to each locus in the reaction mix with the corresponding primers. The annealing temperature used for each locus is shown in table 5. The PCR products were loaded onto a 96 well plate, at both the neat (post-PCR) concentration and at 1/10 dilution, in order to determine which concentration would produce the most legible results. Too low a concentration could produce too faint a peak, whilst too high a peak could distort other results by fluorescing too brightly, causing some light to be given off in other spectra, and producing small false peaks in the analyses of other dye sets. Products from a single sample for each primer set were inserted into the same well, but different samples, primer sets, and concentrations were analysed in separate wells to avoid confusion. 0.5µl of each PCR product or diluted product was added to 10µl HiDi in each well. The plate was sent to the genomics facility at Durham University School of Biological and Biomedical Sciences for fragment analysis using an Applied Biosystems 3730 DNA Analyser, using filter set DS-30, and size standard ROX500, which allows for fragments between 5 and 500 base pairs to be analysed.

The results of that analysis were analysed using Peak Scanner software, and the sizes of each microsatellite locus identified for each of the DNA samples. The size standard was first set to match that used during fragment analysis, then the data were visualised using the software as peaks on a size graph, coloured according to the fluorescent marker used. The size of individual loci were determined for each sample by identifying the peaks that were the correct colour and within the correct size range for that locus, and comparing to the size standard. Up to two peaks were sized for each sample at each locus (the presence of two peaks indicated that the locus was heterozygous), and the sizes recorded.

The results of the genetic analysis were conducted using the GenALEx add-in for excel. The peak sizes determined from the peak scanner software were listed and compared with each other. The potential alleles present were determined by grouping values for different samples at the same locus that fell within 1 base pair of each other in size; different alleles would be at least 2 base pairs apart. The size values were then rounded up or down accordingly, so that all values were listed as integers, in order to be compatible with the GenAlEx formatting requirements. For homozygous loci (for which only one peak was found), the same value needed to be listed twice in the input table, and missing data (which had failed to analyse in peak scanner) were left blank. A pairwise codominant genotypic distance matrix was generated for all samples according to the following rules:

> For a single-locus, with *i*-th, *j*-th, *k*-th and *l*-th different alleles, a set of squared distances is defined as $d2(ii, ii) = 0$, $d2(ij, ij) = 0$, $d2(ii, ij) = 1$, $d2(ij, ik) = 1$, $d2(ij, kl) = 2$, $d2(ii, jk) = 3$, and $d2(ii, jj) = 4$. Genetic distances are summed across loci under the assumption of independence.
> (Peakall and Smouse, 2012, p2.6)

A principal coordinates analysis was then performed, by which the major axes of variation are located within a multidimensional data set, and the axes which reveal the most of the total variation are plotted, in order to visualise the genetic variation in the samples Peakall and Smouse, 2012).

Results

Chelex Extraction method

During the test of the chelex extraction method, 56 of 56 (100%) extractions from single hairs yielded DNA (confirmed by nanodrop analysis- values listed in appendices). A further 4 extractions from single dog hairs collected using hair traps all successfully yielded DNA (Confirmed by presence of fluorescent bands on agarose following amplification and electrophoresis). Extractions generally yielded between 150µl and 200µl of usable supernatant. The amount of DNA produced from a single extraction was therefore estimated to range from 645ng at the absolute minimum, up to 2360ng, based on the maximum variation in both supernatant volume and DNA concentration recorded using the nanodrop machine.

Primer selection

Of the 16 microsatellite primers and one genomic sex-linked primer that were selected for testing, 9 microsatellite loci and the sex marker were successfully amplified in 3 of 3 PCRs at the same annealing temperature. The corresponding primers were selected for use in genetic analysis. Table 3 lists the results of primer selection, and the annealing temperatures that were determined for the reliable primers.

The sex-linked primer, SRY, was tested using the fox samples, and successfully amplified genes 3 times at 56°C from the 2 samples from males, and did not amplify genes from any of the repeats of the female samples, based on electrophoresis gel banding patterns.

Genetic analysis

A total of 108 microsatellites were analysed across 12 samples and 9 loci per sample. Of these, satisfactory size data were obtained from 92. At 3 loci (C02.466, FH2010 and FH2054), data

could not be obtained for all samples, but all samples had data for at least 7 loci. FH2054 had the lowest success rate for size analysis; only 2 fox and 2 dog samples could be analysed at this locus. FH2010 was successfully analysed for 4 fox and 2 dog samples, and C02.466 for 5 foxes and 3 dogs.

The number of alleles present in the samples varied for each locus, and some loci were heterozygous, having different alleles present in the same individual. For loci CXX.374, FH2010 and FH2054, only 1 allele was present in all of the samples. The most variable locus was REN195, which had 9 different alleles present in the samples. In general, loci that were variable in foxes were also variable in dogs, likely because many loci used in fox analysis were first sequenced for use in dogs. A breakdown of the descriptive statistics by population and by locus can be found in table 6.

Table 6: Sample Size, No. Alleles, No. Effective alleles, Information Index, Observed Heterozygosity, Expected Heterozygosity, Unbiased Expected Heterozygosity, and fixation index.

Pop	Locus	N	Na	Ne	I	Ho	He	uHe	F
Fox	**CXX.374**	8	1	1.000	0.000	0.000	0.000	0.000	N/A
	FH2054	2	1	1.000	0.000	0.000	0.000	0.000	N/A
	REN135	8	3	2.327	0.947	0.375	0.570	0.608	0.342
	C17.402	8	2	1.280	0.377	0.250	0.219	0.233	-0.143
	REN195	8	6	4.571	1.630	0.375	0.781	0.833	0.520
	FH2010	4	1	1.000	0.000	0.000	0.000	0.000	N/A
	C27.502	8	3	1.910	0.831	0.625	0.477	0.508	-0.311
	C02.466	7	4	2.513	1.116	0.714	0.602	0.648	-0.186
	AHT142	8	4	2.169	0.987	0.625	0.539	0.575	-0.159
Dog	**CXX.374**	4	1	1.000	0.000	0.000	0.000	0.000	N/A
	FH2054	2	1	1.000	0.000	0.000	0.000	0.000	N/A
	REN135	4	1	1.000	0.000	0.000	0.000	0.000	N/A
	C17.402	4	1	1.000	0.000	0.000	0.000	0.000	N/A
	REN195	4	6	4.571	1.667	1.000	0.781	0.893	-0.280
	FH2010	2	1	1.000	0.000	0.000	0.000	0.000	N/A
	C27.502	4	2	1.600	0.562	0.500	0.375	0.429	-0.333
	C02.466	3	2	1.800	0.637	0.000	0.444	0.533	1.000
	AHT142	4	2	1.280	0.377	0.250	0.219	0.250	-0.143

The principal coordinates analysis explained 60.71% of the variation present in the samples in the first 3 axes, with 27.44% explained by the first axis, 19.32% by the second, and 13.94% by the third. The first two axes are plotted in Figure 7.

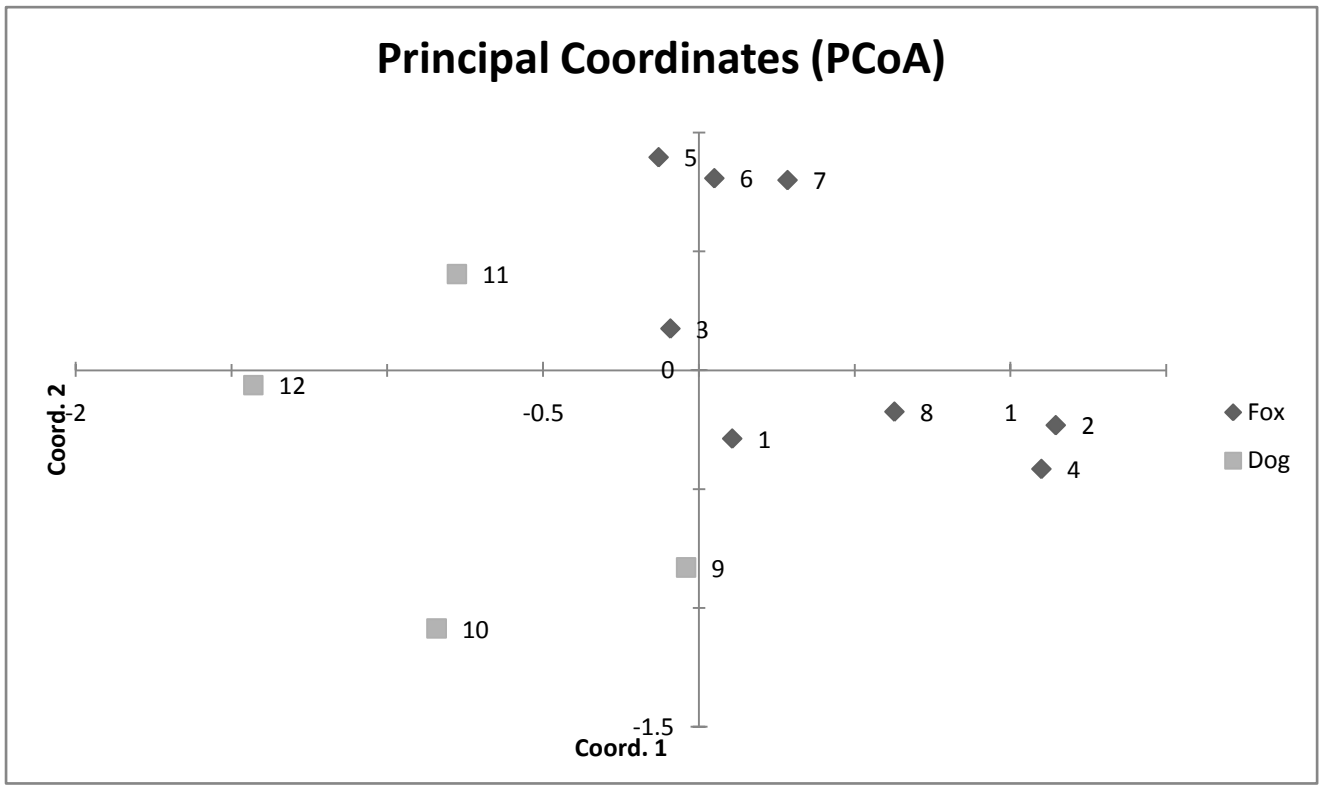

Figure 7: Principal coordinates analysis for all samples. The 2 axes that explained the most variation are shown

Discussion

Despite extensive behavioural studies, the ecology of the red fox remains relatively poorly studied in the United Kingdom (Devenish-Nelson *et al*., 2013), with very few available estimates of local population sizes, and most national estimates relying on relative abundance calculations from records of culled individuals, such as the national game bag census. However in other countries, such as Australia (Berry *et al.*, 2014) and Poland (Mullins *et al.*¸2014), non invasive genetic studies are gaining ground as a means of accurately calculating the size of populations in specific areas, and have the potential to allow more in-depth analysis of population structures and movement patterns.

This study has identified a protocol for the extraction and analysis of DNA from red fox hair samples, which provide a means of recognising individuals and determining their sex from samples as limited as a single hair. These methods could be used in larger-scale future studies of UK red fox populations to develop estimates of population size and genetic diversity.

Extraction Method

The study showed that the chelex extraction method is extremely reliable for extracting DNA from single hairs, making it very useful for use in hair-trapping genetic studies. This could be due to the fact that all of the fox hairs collected for this study were plucked by hand, since plucked hairs are likely to contain follicles, which are the main source of DNA in hair samples (Goossens *et al*., 1998). Hair samples collected from traps are often shed rather than plucked, and may be poorer sources of DNA (a problem encountered by Gonçalves *et al*., 2014). Nevertheless, the results of this study indicate that chelex extraction method could be extremely useful for further studies, and the few samples that were retrieved from traps were all successfully extracted and amplified.

Primers

Primers for 9 out of 14 microsatellite loci were found to amplify red fox DNA successfully with the PCR protocol used, and their annealing temperatures were determined. When analysed together, these loci can be used to distinguish between individual red foxes, as can be seen in figure 1, providing a means of performing studies that require individual recognition, such as capture-mark-recapture analysis. The primers for the sex-linked marker also worked reliably, and can be used alongside the microsatellite markers to enable more in-depth male- and female-specific population analysis from hair samples.

The microsatellite loci varied in how much information they provided about the population, with 3 monomorphic loci (FH2054, FH2010 and CXX.374) having only 1 allele expressed in all 12 samples. This unfortunately coincided in FH2054 and FH2010 loci with high failure rates of the fragment analysis, which further reduced the already small sample size for these markers. Successful genotyping of larger samples might lead to the observation of more alleles for these loci. However, only one allele was detected at locus CXX.374, despite data being obtained from all of the samples. This apparent lack of variation contrasts with the results of Berry *et al*. (2012), who found this marker to be sufficiently variable to be useful in studying Australian red foxes, with a probability of identity of 0.1596 for unrelated individuals and 0.454 for full siblings . Given that the Australian population is less genetically variable than European populations, having undergone a relatively recent population bottleneck (Statham *et al*., 2014), this may be due to post-bottleneck genetic drift in the Australian population. These sorts of events are likely to lead to different allele profiles in UK and Australian fox populations, which is why a different set of primers may be required to perform reliable individual identification in UK populations. The finding may also simply be a result of the small sample size in this study, as these samples were all taken from the same farm, and could have included related individuals. If so, this locus may still prove useful for genetic analysis but it still needs to be tested in larger samples.

The sequences at loci REN195, AHT142 and C02.466 were much more variable, with 9, 5 and 4 alleles, respectively, among the 12 samples. For the fox population, the markers at REN195, C02.466, and AHT142 had the highest information indices, at 1.63, 1.116, and 0.987, respectively, which suggests that these could be very useful for distinguishing between individuals. These results were partially consistent with Berry *et al*. (2012), who record AHT142 as having the lowest probability of identities among unrelated and full sibling samples of the primers that they used.

The study also suggests that the red fox and dog populations were genetically distinct; following principal coordinate analysis, the two populations could be distinguished by the Eigen values of the first two axes. Although the sample size was small, especially for dogs, there is potential for this difference to be used to distinguish dog samples from fox samples by their principal coordinates. For example, samples with negative values for 1st and 2nd axis coordinates could reasonably confidently be identified as dog samples based on these results; in particular, the markers REN13, C02.466 and AHT142 seemed to have the most variation between the two populations, based on the differences in allele frequencies by population. With further study, it could be possible to develop a frame of reference against which samples could be checked to determine whether they were taken from dogs or foxes.

This has important implications for the study of red foxes in the UK, as domestic dogs are the most closely-related species present in this country. Hairs of the two species can generally be distinguished visually, with the aid of a microscope, but smaller hairs may be harder to identify, and without the use of additional genetic species identification analyses, there is a risk that dog hairs may be erroneously identified and analysed as fox samples. If it is possible to identify such mistakes following analysis then it may not be necessary to include additional species identification procedures in UK red fox studies, which would potentially save time and money, making the procedure more efficient.

Conclusions

This study has confirmed that the Chelex methods is very reliable for the extraction of DNA from remotely-collected single-hair samples. A set of microsatellites have been found which can be successfully and reliably used to distinguish individual red fox samples from each other, and a sex-linked marker which was found to accurately distinguish male samples from females. Furthermore, the microsatellites were found to differ sufficiently between dogs and foxes to suggest that they could be of use in identifying non-target DNA samples, should visual species identification fail. These

methods can be used in future larger-scale studies of red foxes, in order to further our understanding of UK fox populations.

Chapter 4: General Discussion

The red fox (*Vulpes vulpes*) is an important natural predator in the UK, and is widely managed. However, the monitoring of UK fox populations relies heavily on index sampling techniques such as game-bag censuses (Newsome *et al.,* 2010), faecal counts (Webbon *et al*., 2004) and spotlight counts (Baker and Harris, 2006). The use of such index techniques in ecology is the subject of contention, as there is a high level of uncertainty around the relationship between indices of abundance and true abundance (Stephens *et al*., 2015). This can be especially problematic when these estimates are used to set management objectives and monitor culling success; measures of true abundance enable greater certainty in these tasks, and can be used to explore the relationship between true abundance and indices. Consequently, a useful and reliable method of estimating true abundance for UK fox populations would improve our ability to monitor and manage them.

Non-invasive Genetic Sampling (NGS) has shown great potential recently as a tool for estimating the true abundance of mammal populations (Piggott and Taylor, 2003), and has been used successfully to monitor the response of Australian fox populations to lethal control (Berry *et al*., 2012; 2014). These techniques therefore have great potential to improve UK red fox monitoring, but had not hitherto been tested for this purpose, and it was not known how high levels of rainfall, dense woodland environments, and interference from competitors such as dogs and badgers would affect them. Furthermore, UK populations are genetically isolated from Australian and European foxes, and genetic methods needed to be re-evaluated using UK samples to ensure that they could reliably be used.

The research presented in previous chapters aimed to evaluate the effectiveness and feasibility of using NGS to monitor foxes in Durham City woodland by performing genetic analysis of remotely collected hair samples. This was divided into two parts. Firstly, several trap designs, incorporating different hair collection structures and baits, were trialled for their effectiveness in attracting foxes and collecting hair samples. Secondly, a set of test samples of hairs collected from culled UK foxes were used to test the reliability of DNA extraction using Chelex, test a range of microsatellite primers in order to develop a set that could be used for individual recognition of samples from UK fox populations. Hairs collected from domestic dogs were also tested to determine how interference from dog samples might affect this analysis, and whether the samples could be distinguished from fox samples if some were mistakenly analysed.

During the hair trapping trials, none of the trap designs successfully collected any fox hairs. Meat baited traps collected hairs from non-target organisms that took the baits, especially badgers

and dogs, and corvids were also found to regularly take meat baits. This interference is likely to have reduced the effectiveness of the traps. Despite being successfully used to attract foxes in other studies (e.g. Monterroso *et al*., 2014), valerian-scented traps did not collect any hairs from any organisms.

The laboratory techniques showed much greater potential for use in future studies. The Chelex extraction method was found to be extremely reliable in extracting DNA from single-hair samples, which allows it to be confidently used for genetic analysis of extracted hairs. It is likely that it would experience a lower success rate in practice, as remotely-collected samples often contain a lot of shed hairs, which are less likely to yield DNA than the plucked hairs used in these tests (Monterroso *et al*., 2012). However, these results show that the Chelex extraction method can be relied upon to extract DNA when it is present. With good sample-collection practice, including regular sample collection and careful storage to prevent DNA degradation, the chances of collected hairs containing usable DNA can be quite high (Piggott and Taylor, 2003; Sloane *et al.,* 2000), and the Chelex extraction method will be more than sufficient.

A set of microsatellite markers was also established in this study which was successfully used to differentiate and identify the sex of individuals from a single UK fox population. These markers could be used in future genetic analysis of red foxes, and with suitable hair-collection methods, could be used to produce non-invasive capture-mark-recapture estimates of UK fox abundance. Additionally, tests of non-invasively collected dog samples showed that they were sufficiently genetically distinct from fox samples to be separated following genetic analysis. This has important implications for non-invasive genetic analyses of fox hairs, as the ability to confidently distinguish samples following analysis, when combined with visual analysis, could reduce the need for separate genetic species identification steps. Since the quantity of DNA collected from a single hair is often very small, removing these additional steps will leave more available to be amplified for the primary analysis, and reduce errors that arise from limited genetic material.

The difficulties with attracting foxes to the hair traps in this study are likely not insurmountable, as studies of other fox populations have been able to draw them to traps. The positioning of the traps in this study was not ideal, as they were away from paths, which may have contributed to increased interference from badgers, and resulted in fewer fox visits. Future studies may have greater success with traps placed closer to paths, although they will risk greater interference from humans and domestic dogs.

There are also alternative approaches which could be used to provide absolute abundance estimates for fox populations. In particular, scat samples could be a potential alternative to hairs.

They can also be collected non-invasively, and can be analysed using the majority of the same methods, with some alterations to the Chelex extraction method (Piggott and Taylor, 2003). Like hairs, scats can also be visually identified, and scat counts have already been used in the UK to produce index counts (Webbon *et al*., 2004). With the addition of the microsatellite analysis methods refined in this study, they could form the basis for NGS studies that could improve the current state of UK fox monitoring.

Scat surveys could circumvent the issue of attracting foxes to traps, as they do not need to draw foxes to a specific area. However, As sources of DNA, scats are more problematic than hairs, as they contain DNA from multiple prey species in addition to that of the target organism, and are therefore associated with a much higher risk of contamination, requiring more costly extraction processes to be used (Amendola-Pimenta *et al*., 2009; Waits and Paetkau, 2005).

Scats can also be hard to locate visually, and scat surveys generally require the use of trained scat detection dogs. This enables samples to be collected relatively quickly, and has higher sample detection rates than hair sampling (Vine *et al*., 2009), but is costly (Clare *et al*., 2015), especially once the cost of genetic analysis is factored in. It may also be difficult to collect enough samples for analysis in areas with low population density, since sample collection is dependent on the scat deposition rate. Consequently, NGS studies that rely on scat samples are likely to be most effective for large fox populations, or when performed over longer time-scales, to ensure that large numbers of samples are collected.

The use of scat detection dogs is vulnerable to some of the same problems that hamper scent lures. In particular, the inhibition of the dispersal of scat scents in complex environments can increase the risk of scats being missed (Leigh and Dominick, 2015), which could result in smaller sample sizes or even type II errors, in which foxes are incorrectly thought to be absent. Increased survey effort would be required in such environments in order to overcome this issue, and would drive up the cost still further. Consequently, the increased cost over index sampling is likely to limit the scale and frequency of NGS studies of UK foxes that can be conducted if they need to rely on scat samples.

These are all issues that can be overcome, and NGS studies of fox scats can be useful (e.g. Vine *et al*., 2009). Webbon *et al*. (2004) found that scat counts could be used to estimate UK fox density over large spatial scales, which indicates that, over these scales, it is possible to find large numbers of fox scats even without the use of scat detection dogs. At these scales, therefore, it is likely that scat collection could be a viable method of gathering samples for non-invasive genetic population analysis. Such studies could be used to monitor changes in red fox populations at

regional and even nationwide scales in the UK, which are currently performed very infrequently (Webbon *et al*., 2004). Even if local-scale studies remain impractical, these estimates could greatly improve the state of UK fox population monitoring, and allow management decisions to be made with greater confidence.

Another approach that could improve our understanding of fox population structures and movement dynamics, though without necessarily improving the accuracy of abundance estimates, is to apply the genetic analysis approach to samples taken from foxes that are killed as part of management practice. Blood or tissue samples provide higher quality DNA than non-invasive methods (Piggott and Taylor, 2003), and the analysis of such samples can provide information about fox dispersal patterns which could inform management decisions. A recent large-scale study by Statham *et al*. (2014) analysed DNA from red fox tissue samples to gather information about historical range-wide dispersal and genomic exchange; such studies can also be performed between populations on a much smaller scale, providing information about local dispersal and breeding patterns, which could enable researchers to better understand the way that foxes respond to local control efforts, leading to the improvement of management schemes. When undertaking these kind of studies, tissue taken from road kill may also be used to provide additional samples; as long as it is collected relatively quickly, decomposition is not too big an issue, and it has proved a useful source of additional data in some cases (e.g. Statham *et al*., 2014).

Whilst this approach obviously prevents these samples from being of use for non-invasive capture-mark-recapture analyses, research by Waples and Do (2009) into single-sample population estimates suggests that there is still a possibility that the effective size of the population from which individuals were culled could be estimated without the need for repeat sampling, using a linkage disequilibrium approach. This could also be performed using the microsatellite marker set developed in this study, although a few more markers would be required to make accurate estimates (10-20 are recommended; Waples and Do, 2009). The precision of the estimates produced using this method varied with population size; for effective population sizes of 500-1000 and above, much larger sample sizes would be required to maintain precision. However, the effect of doubling the number of loci analysed had the same effect as doubling the sample size, which could be used to overcome issues with sample size if this approach was applied to samples from culled foxes.

Although the linkage disequilibrium approach has limited applications for monitoring responses to management, it could be used alongside population indices estimated from culling or hunting takes, to provide information on the relationship between the index and effective population size. Ultimately, this information could be used to calibrate and improve population size estimates from surveys such as the National Game Bag Census (Newsome *et al*.,2010).

Although the traps tested in this study were not able to collect fox hairs, they did successfully collect hairs from other UK fauna. In particular, the sticky wicket traps collected 64 hair samples over 200 trap nights, and of these, 47 samples were from badgers. Like the red fox, European badgers (*Meles meles*) are an important and widely managed UK predator species. Badgers are also well-know transmitters of infection, including bovine tuberculosis (TB), and badger culls as a means of controlling the spread of TB are the topic of much controversy (Donnelly *et al*., 2015). It is thought that the effectiveness of such culls may be compromised by changes in badger behaviour, such as increased movement following culls, which could spread TB between populations (Bielby *et al*., 2014). Genetic identification studies of badger populations in and around culling sites could provide an additional means of monitoring the effect of culling on population size and individual movements, which could bring useful information to the culling debate; the sticky wicket design used in this study would allow genetic samples to be collected pre-culling without risking perturbation that might be caused by live-capture studies.

Irrespective of the species studied, however, non-invasive genetic analysis has great potential for improving ecological study by expanding the genetic analysis stage of remote-sampling studies to produce measures of genetic diversity as well as population size. This would allow for much more in-depth monitoring of the populations, and could pick up on important population effects that might be missed by simply monitoring population size, and improve monitoring of population demographics, such as effective population size (Luikart *et* al., 2003; Schwartz *et al*., 2007).

Genetic information gathered from remotely collected samples could also be mapped alongside location data, which would allow monitoring of gene flow in target populations (Manel *et al.*, 2003). This approach could distinguish between individuals recruited to the population through reproduction, and through migration from other populations. Such information could be very useful when monitoring management efforts on species such as foxes and badgers, which may replenish populations through migration following culls (Bielby *et al*., 2014; Baker and Harris, 2006).

Conclusions

There remains a need for improvements to the state of red fox population monitoring in the UK. Non-invasive genetic sampling (NGS) has the potential to produce accurate population estimates without affecting the population being monitored. This study has successfully developed a protocol for extracting and analysing DNA from remotely collected hair samples, for use in producing capture-mark-recapture estimates of true abundance in UK fox populations. This could be extremely useful for future studies of UK fox populations, as it may significantly reduce the amount of time and

money that need to be spent on researching and developing protocols suitable for the target population. These costs are one of disadvantages of using new tools in ecological studies, and it is hoped that the findings of this study could allow future research to take place using non-invasive genetic techniques, which could lead to improvements in our understanding of red fox population dynamics in the UK.

In order for NGS to be used to successfully monitor UK red foxes, future research should focus on developing a suitable method of collecting samples for use with the developed laboratory protocols. This could either involve further trials of hair-collection traps that could be used with the protocols developed in this study, or investigating the feasibility of collecting scat samples for genetic analysis. Research into the use of scat samples should consider the cost and efficacy of scat detection dogs as a sample collection tool, especially in complex woodland environments. The extraction method trialled in this study will also need to be modified for use with scat samples, in order to overcome the problems with high levels of contamination.

The increased cost of scat detection dogs over hair collection traps could be prohibitive; hair traps still have a lot of potential as a cost effective NGS tool. Future research into the use of remote hair collection to monitor foxes could develop the findings of this study. The 'sticky wicket' trap design was the most successful at collecting hair samples from mammals that were attracted to them, and has the most potential of the traps tested. In order to overcome the issues with attracting foxes to the traps, these traps could be tested with different baits, to determine a bait type which can successfully draw. Adding sugar or beef stock to food baits was found by Saunders and Harris (2000) to improve their attractiveness to foxes, and could be used with the sticky wicket traps. Future trials could also use fox-based scents after setting traps and removing hairs in order to cover up the evidence of human activity, in order to determine whether lingering human scent was preventing foxes from entering the traps.

Another useful focus for future research could be to build on the findings of the laboratory research in this study, by establishing a framework for distinguishing dog and fox samples. This could be done by analysing a large number of samples from each species, and developing a database against which outlying samples in future studies could be compared, in order to find and exclude non-target dog samples.

Whilst there is still a need for increased monitoring of UK red fox populations using tried and tested index sampling methods, in order to improve our understanding of the species' movement patterns and response to control, by focusing on developing NGS methods, future research could

allow more accurate estimates of fox abundance to be produced, which could be used to reduce uncertainty in red fox management.

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